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| (54) Title: APOPTOSIS-RELATED COMPOUNDS AND THEIR USE | | | |
| (57) Abstract Apoptosis-related antigenic compounds comprising an exposed antigenic site having the amino acid sequence SEQ ID NO:1: (A) or a functionally equivalent sequence comprising at least the sequence Ala Leu Asp are disclosed. Further, several applications and uses based on such compounds are included, such as use of the compounds in medicaments, nucleic acid sequences encoding the amino-acid sequence of such a peptide compound or protein fragment comprising the C-terminal amino acid sequence Ala Leu Asp, anti-sense nucleic acid sequence, vector comprising such a nucleic acid sequence, antibody or antigen-binding peptide recognizing such an antigenic compound and use thereof in a medicament, agents regulating the liberation of protein fragments comprising the amino-acid sequence SEQ ID NO:1 or functionally equivalent sequences comprising the C-terminal amino acid sequence Ala Leu Asp, a method of determining the occurrence of cell apoptosis in a sample of biological material, especially in the diagnosis of degenerative diseases and cancer, or monitoring of the effect of therapy, a method of treating diseases with involvement of apoptosis, such as cancer and degenerative diseases, a method of creating cancer cells, and as carrier for prophylactic, therapeutic or diagnostic use. | | | |

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APOPTOSIS-RELATED COMPOUNDS AND THEIR USE

- 5 The present invention relates to apoptosis-related antigenic compounds, such as fragments of cytokeratin 18, and antibodies or antigen-binding peptides recognizing said compounds, which are useful in medicaments, diagnostics and methods for the detection, monitoring, measurement and regulation of the type of cell death called programmed cell death or apoptosis. The determination
10 of the occurrence of cell apoptosis may further be used for the monitoring of the effect of therapeutic treatment.

Background of the invention

- 15 Apoptosis is seen in all sorts of higher eucaryocytes from plants and insects to vertebrates (Kroemer G, Petit P, Zanzami N, Vayssiere J-L, Mignotte B: The biochemistry of programmed cell death. The FASEB Journal 9:1277-1287, (1995)). Apoptosis is a general phenomenon of vital importance. Decreased apoptosis leads to malformation, cancer and autoimmune disease, and
20 enhanced apoptosis results in degenerative diseases, acute diseases such as infection by toxin-producing microorganisms and in rejection of transplanted organs.

- Therefore, the detection, monitoring, measurement and regulation of apoptosis
25 are important factors in the diagnosis and therapeutic treatment of the mentioned conditions.

- Recently, Caulin C., et al (The Journal of Cell Biology, Vol 138, pp. 1379 -1394) studied caspase cleavage of Keratin 18 and reorganization of intermediate
30 filaments during epithelial cell apoptosis. Keratin 18 was cleaved in vitro by caspase-6, -3, and -7, and it was stated that the cleavage site common for the three caspases was the sequence VEVD/A, located in the conserved L1-2 linker region of K18.

D scription of the inv ntion

During research work aiming at finding new useful specific monoclonal
5 antibodies reacting with human cytokeratin 18 (CK 18), in addition to those
already at hand, it was surprisingly found that a monoclonal antibody (MAb)
(obtained after immunization of mice with a specified part of the amino-acid
sequence of CK 18) recognized early apoptotic changes in cultured epithelial
cells. This MAb was designated M30.

10

A detailed analysis, including synthesis and assay of a large number of amino-
acid (aa) sequences revealed, that the specific epitope and binding-site for the
MAb M30 consists of the aa sequence: EDFNLGDALD. This 10 aa peptide
sequence starts from the second coil of human CK 18 and represents aa
15 No.383-392. This specific epitope is a neo-epitope liberated during apoptis
and it is not exposed in the intact CK 18 molecule. The complete aa sequence
of CK 18 was published by Oshima et al. (Oshima RG, Millan JL, and Ceccena
G. (1986). Comparison of mouse and human keratin 18: a component of
intermediate filaments expressed prior to implantation. Differentiation 33:61-
20 68.).

The different aspects of the present invention are based on the optimal binding
site of the mAb M 30, i.e. the amino-acid sequence EDFNLGDALD,
SEQ ID NO: 1:

25 Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp
1 5 10

or functionally equivalent sequences comprising the sequence Ala Leu Asp.

Initially it was believed that an important part for apoptosis is related to cleavage
30 by caspase enzymes immediately after DALD (Asp Ala Leu Asp), since this
sequence occurs in CK18 and caspases are known to cleave after DXXD
wherein X stands for not defined aa.

However, the sequence DALD does not occur in such other intermediate filaments as CK 1- 8, vimentin, desmin, different actins, neurofilaments, and lamins. In these the following sequences are found: LALD, MALD, MALD, VALD, MALD, AND LALD, respectively.

5

Since the mAb M 30 recognizes apoptotic changes in non-epithelial cells such as cardiac muscle, mega- karyocytes, myeloblasts and neural tissues of fetus - which are not known to contain CK18 - it is now believed that detection of protein fragments having the C-terminal sequence ALD indicates early apoptosis.

10

Thus, one aspect of the invention is directed to an apoptosis-related antigenic compound which specifically binds to an antibody which in turn specifically binds to the amino-acid sequence

15 SEQ ID NO: 1:

Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp

1

5

10 ,

i.e. an apoptosis-related antigenic compound comprising an exposed antigenic site having the amino acid sequence SEQ ID NO: 1 or a functionally equivalent sequence. Further, the functionally equivalent sequence should comprise at least the sequence Ala Leu Asp.

20

It is the three-dimensional structure of the exposed antigenic site, or antibody-binding site, that defines the structure of the binding portion of an antigenic compound which specifically binds to the corresponding three-dimensional structure of an antibody. The antigenic compound of the invention therefore comprises compounds which are based on the structure of the SEQ ID NO:1, i.e. the sequence as such or a functionally equivalent sequence, i.e. homologous in function, comprising at least the sequence Ala Leu Asp, such as compounds having replacements of one or several amino acids in the above specified amino-acid sequence with other molecular parts, D- forms of the amino acids, non-natural amino acids and/or derivatives, as long as the three-dimensional structure of the SEQ ID NO:1 is mimicked. Therefore the common

30

feature of the antigenic compounds of the invention is that they bind specifically to an antibody which in turn binds specifically to the aa sequence SEQ ID NO:1, i.e. antigenic compounds comprising an antigenic site having the amino acid sequence SEQ ID NO: 1 or a functionally equivalent sequence comprising at least the sequence Ala Leu Asp.

It can be mentioned that intact CK 18 molecules have the above-specified sequence hidden, and can therefore not react with antibodies specifically binding to said sequence, thereby making the recognition specific to apoptosis, i.e. those cases where the CK 18 molecule has been already cleaved after the C-terminal Asp of the sequence Asp Ala Leu Asp.

In a preferred embodiment of this aspect of the invention, the apoptosis-related compound is a peptide or a protein fragment comprising the C-terminal amino acid sequence Ala Leu Asp. The peptide may be a homologue to, an extension of or a truncation of the SEQ ID NO: 1, i.e. may have a homologous sequence having some amino-acid substitutions, extensions, truncations and/or deletions which do not lead to the elimination of the capability of the peptide to bind to the same antibodies as the amino-acid sequence SEQ ID NO: 1.

In another preferred embodiment of this aspect of the invention the antigenic compound of the invention is a peptide or a protein fragment comprising the C-terminal amino acid sequence Xaa Ala Leu Asp, wherein Xaa is selected from Asp, Leu, Met, and Val.

An example of a peptide according to the invention is an oligopeptide having the amino-acid sequence

SEQ ID NO: 1:

Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp

1

5

10

Another example is an oligopeptide having the amino-acid sequence (EDGEDFNLGDALDSSNSMQT)(i.e. SEQ ID NO: 1 extended by 3 amino acids at the N-terminal and 7 at the C-terminal)

5 SEQ ID NO: 2:

| | | | | | | | | | | | | | | | |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Glu | Asp | Gly | Glu | Asp | Phe | Asn | Leu | Gly | Asp | Ala | Leu | Asp | Ser | Ser | Asn |
| 1 | | | | 5 | | | | | 10 | | | | | 15 | |
| Ser Met Gln Thr | | | | | | | | | | | | | | | |
| 20 | | | | | | | | | | | | | | | |

10

Yet another example is an oligopeptide having the amino-acid sequence (LEDGEDFNLGDALDSSNS)

SEQ ID NO: 3:

| | | | | | | | | | | | | | | | | |
|----|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 15 | Leu | Glu | Asp | Gly | Glu | Asp | Phe | Asn | Leu | Gly | Asp | Ala | Leu | Asp | Ser | Ser |
| | 1 | | | | 5 | | | | | 10 | | | | | 15 | |
| | Asn Ser . | | | | | | | | | | | | | | | |

20 These peptides having the amino acid sequences SEQ ID NO: 2 and 3 have been synthesized, and in an ELISA they bind to the mAb M 30 [relative activity : 100 for SEQ ID NO: 1; 11 for SEQ ID NO: 2; and 8.3 for SEQ ID NO: 3, respectively]. However, these two peptides are not expected to represent cleaved fragments of CK18 specific for apoptosis, since the mAb M 30 cannot bind to CK18 fragments until they expose the neo-epitope having the C-terminal

25 sequence Ala Leu Asp.

Still another example is an oligopeptide having the amino-acid sequence (GEDFNLGDALD)

SEQ ID NO: 4:

30

| | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Glu | Asp | Phe | Asn | Leu | Gly | Asp | Ala | Leu | Asp |
| 1 | | | | 5 | | | | | 10 | |

In the present specification and claims, specific binding of an antigenic

35 compound to an antibody, or specific binding of an antibody to an amino-acid

sequence, requires an affinity constant of at least 10^7 liters/mole, preferably at least 10^9 liters/mole.

In an embodiment of this aspect of the invention the antigenic compound is
5 coupled to a carrier and/or label.

The carrier may be e.g. plastic surfaces, such as microplates, beads etc.; organic molecules such as biotin; proteins, such as bovine serum albumin; peptide linkers, polypeptides e.g. resulting in fusion proteins.

10 The label may be selected from a variety of different types of labels, such as radioactive isotopes, enzymes, fluorescent markers, etc.

Another aspect of the invention is directed to an antigenic compound according to the invention for use in a medicament.

15

In a preferred embodiment of this aspect of the invention an antigenic compound according to the invention is used in the production of a medicament for inhibition of cell apoptosis.

20 Inhibition of cell apoptosis may be desirable in the treatment of degenerative conditions, such as anorexia, AIDS, transplantation of organs, psoriasis, and Alzheimer's disease.

Yet another aspect of the invention is directed to an isolated or recombinant
25 nucleic acid sequence comprising a nucleotide sequence which encodes an amino-acid sequence of a peptide or protein fragment according to the invention.

A nucleic acid sequence of the present invention may be either in the form of
30 RNA or DNA (including cDNA, genomic DNA and synthetic DNA). The DNA may be double-stranded or single-stranded. When the DNA sequence is single-stranded, it may be either the coding sequence (sense strand) or non-coding sequence (anti-sense strand). The nucleic acid sequences of the invention can

be designed from the amino-acid sequence of a peptide or protein fragment comprised by the invention with the aid of the genetic code.

The nucleic acid sequences of the invention can be used in the production of proteins, polypeptides, oligopeptides and peptides of the invention.

5 In a particular embodiment of this aspect of the invention, the nucleic acid sequence is an anti-sense sequence based on the nucleic acid sequence of the invention, which is complementary, in whole or at least a part of the SEQ ID NO: 1 encoding part, to a sense sequence encoding a peptide or a protein fragment

10 of the invention. When introduced into a cell, the anti-sense nucleic acid sequence can inhibit the expression of the gene encoded by the sense strand.

This aspect of the invention also involves a vector comprising a nucleic acid sequence of the invention. Such a vector may be used in the production of a peptide or protein fragment of the invention or possibly in the regulation of the

15 amount of protein fragments comprising the amino acid sequence SEQ ID NO: 1 or functional equivalents thereof comprising at least the sequence Ala Leu Asp in target cells, *in vivo* or *in vitro*. In a preferred embodiment of the invention, the vector is a plasmid.

20 Still another aspect of the invention is directed to an antibody or antigen-binding peptide recognizing an antigenic compound according to the invention.

The antibody of the invention may be a monoclonal antibody or monospecific

25 polyclonal antibody recognizing or specifically binding to an antigenic compound according to the invention, i. e. recognizing or specifically binding to the amino-acid sequence SEQ ID NO: 1 or a functionally equivalent sequence comprising at least the sequence Ala Leu Asp. The antibody of the invention can be prepared by using immunization procedures well known in the art, or by well

30 known methods based on recombinant technology making use of suitable host cells of eukaryotic or prokaryotic origin.

The antigen-binding peptide recognizing an antigenic compound according to the invention may be the antigen-binding part of an antibody, or a protein or peptide which has an amino-acid sequence that at least in the part corresponds to the amino-acid sequence SEQ ID NO: 1 or its functional equivalent comprising at least the sequence Ala Leu Asp but has amino acids with the opposite charges. Such an antigen-binding peptide will function as an antagonist.

In the development of antagonists the following guideline may be used:

10 o = hydrophobic amino acid

+ = positive amino acid

- = negative amino acid

± = neutral amino acid

15 SEQ ID NO: 1 : E D F N L G D A L D

- - o ± o ± - o o -

corresponding site

of antagonist : + + o ± o ± + o o +

e.g. K R W Q F G R L A R

20 A number of antagonist candidates may be designed with the aid of the above guideline, especially variants where K and R are used for the + positions.

In an embodiment of this aspect of the invention the antibody or antigen-binding peptide is coupled to a carrier and/or label.

25

The carrier may be e.g. plastic surfaces, such as microplates, beads etc.; organic molecules such as biotin; proteins, such as bovine serum albumin; peptide linkers, polypeptides e.g. resulting in fusion proteins.

30 The label may be selected from a variety of different types of labels, such as radioactive isotopes, enzymes, fluorescent markers, etc.

A further aspect of the invention is directed to the antibody and antigen-binding peptide of the invention for use in a medicament.

- In a preferred embodiment of this aspect of the invention an antibody or antigen-binding peptide of the invention is used in the production of a medicament for the stimulation of apoptosis, particularly in conditions such as malformation, cancer and autoimmune disease, i.e. conditions relating to uncontrolled or excessive cell proliferation.
- 10 In another embodiment of the invention the produced medicament is desirable in the treatment of diseases with involvement of enhanced apoptosis, such as many degenerative diseases, or acute diseases such as infection by toxin-producing microorganisms or ischemic-reperfusion damage and rejection of transplanted tissue or organs. Here, medical control of apoptosis may be of utmost importance.

- Yet another aspect of the invention is directed to agents regulating the liberation in biological material, including the mammalian body or cell culture, of protein fragments comprising the C-terminal amino-acid sequence
- 20 Ala Leu Asp .

- Examples of such agents of the invention are nucleic acid sequences of the invention, either inhibiting (sense strand) or stimulating (anti-sense strand), the expression of proteins comprising the sequence SEQ ID NO: 1 or sequences specifically binding to the same antibodies or antigen-binding peptides as the SEQ ID NO: 1, i.e. functionally equivalent sequences comprising at least the sequence Ala Leu Asp, and enzymes, enzyme activators and inhibitors.

- Still another aspect of the invention is directed to a method of determining the occurrence of cell apoptosis in a biological sample including a sample of an organ, tissue or body fluid from a mammal, including man, wherein the presence of protein fragments comprising the C-terminal amino-acid sequence Ala Leu Asp, is determined.

In a preferred embodiment of this aspect of the invention the determination is performed with an immunological assay using the antibody of the invention.

- 5 In another preferred embodiment of this method the rate of occurrence of cell apoptosis is determined. The determined rate of cell apoptosis may be used in the diagnosis of diseases with involvement of apoptosis, such as degenerative diseases and cancer, and in the monitoring of the effect of therapy.
- 10 As origin of samples in this aspect the following may be mentioned: in vivo or in vitro, any organs, normal or changed, tissues, specimens and fluids of the human or animal body; for example liver, lung, kidney, heart, spleen, brain, viscera, lymphatic organs, bone-marrow, reproductive organs, skeleton, muscle, skin, sensory organs, glands, blood, serum, urine, ascitic fluid, pleural fluid,
- 15 cerebrospinal fluid, amniotic fluid, abscess fluid, wash fluids, punctures, slices and any preparation of cellular or fluid origin in this context.

- The determination of said protein fragments may be performed by any technique which can detect and preferably quantify the amount of peptide
- 20 fragments comprising the C-terminal amino acid sequence Ala Leu Asp in a sample of body fluid. Preferably the protein fragments comprise at least the amino acid sequence SEQ ID NO: 1 or sequences specifically binding to the same antibodies or antigen-binding peptides as the SEQ ID NO: 1, i.e. functionally equivalent sequences comprising at least the sequence Ala Leu
- 25 Asp. Monoclonal antibodies which specifically bind to said fragments can be used in different immunoassays, optionally labeled in accordance with the actual assay used.

- In one embodiment of said method of the invention, the determination is
- 30 performed with the aid of an immunoassay. Also, detection systems adapted for the use of antibody-site carrying structures and/or antibodies may be used. Some examples of the numerous immunoassays which may be used in the invention are Enzyme-linked immunosorbent assay (ELISA),

Radioimmunoassay (RIA, IRMA), Fluorescence immunoassay (FIA), Chemiluminescent enzyme-labeled immunometric assay, Luminescence immunoassay (LIA), Dissociation enhancement time-resolved fluoroimmunoassay (DELFLIA). The assay may be manual or automatic.

5

Further, an aspect of the invention is directed to a method of treating diseases with involvement of apoptosis, such as cancer and degenerative diseases, in a patient comprising administration of a cell apoptosis-regulating amount of an antibody or antigen-binding peptide according to the invention or of an antigenic compound according to the invention, to said patient.

10

Another aspect of the invention is directed to a method of creating cancer cells comprising administration of a cell apoptosis-inhibiting amount of an antigenic compound according to the invention to a cell culture or a laboratory animal.

15 Such a cell culture or laboratory animal can be used in the production of desired polypeptides or proteins or in the evaluation of candidate anti-cancer drugs.

An additional aspect of the invention is directed to a carrier for prophylactic, therapeutic or diagnostic use comprising an antibody or antigen-binding peptide of the invention. Such a carrier of the invention will function as a targeting substance finding fragments comprising the C-terminal amino acid sequence Ala Leu Asp, such as SEQ ID NO: 1 or functionally equivalent sequences comprising at least the sequence Ala Leu Asp, and it may be coupled to a variety of medicaments and/or labels for prophylactic, therapeutic or diagnostic purposes.

20
25

The present invention will now be further illustrated by reference to the following description of experiments and specific embodiments of the invention, which are not to be considered as limitations to the scope of the invention defined in the claims.

30

Synthesis of peptides of the invention

The peptides of the invention can be produced by any known method of producing an amino-acid sequence, such as, controlled degradation of a purified protein by proteases or other chemical methods (Allen G., Sequencing of proteins and peptides, 1989, Elsevier Science Publishers B.V.). Chemical synthesis is commonly performed by coupling of the amino acid residues or peptide fragments to one another in correct order in liquid phase to produce the desired peptide. Another common strategy is the coupling of the amino acids to one another starting with a solid phase (resin) to which the C-terminal of the last amino acid of the sequence is coupled, whereupon the C-terminal of the penultimate amino acid is coupled to the N-terminal of the last amino acid, etc., finally releasing the built-up peptide from the solid phase (so called solid-phase technique).

15

The oligopeptides made in order to illustrate the invention were synthesized using the multipin peptide synthesis approach using polyethylene supports derivatized with an acid handle (Cf. Valerio, R.M., Bray, A.M. and Maeji, N.J. (1994) Multiple peptide synthesis on acid-labile handle derivatized polyethylene supports. Int. J. Peptide Protein Res. 44:158-165.). The synthesis was carried out on detachable pins grafted with hydroxyethylmethacrylate and functionalized with the trifluoroacetic acid-labile Rink amide forming handle. Peptides representing both the N and C terminal truncation series of the sequence EDFNLGDALD and other sequences were synthesized. Peptides were capped with biotin using the tetra peptide linker sequence -SGSG- or -SGSB- (B = β alanine), and attached to streptavidin coated plates in the enzyme-linked immunosorbent assay.

The aa sequences were checked for purity by reverse phase high performance liquid chromatography (RP-HPLC) and by ion spray mass spectrometry (IS-MS) (Cf. Van Dorsselaar et al., (1990) Application of electrospray mass spectrometry to characterization of recombinant proteins up to 44 kDa. Biomed. Environ. Mass Spectrom. 19:692-704).

Ion spectra were collected as positive ions, obtained by vaporizing the peptide from acidic (trifluoroacetic or acetic acid) solution. Percentage of total ion count versus molecular weight (Dalton) was derived from the spectrum of raw mass-to-charge ratio versus ion count. The IS-MS purity value is the ion count, attributable to the target peptide, expressed as percentage of the total ion count.

Immunization of mice

10 Preparation of immunizing material

The starting material for antigen purification consisted of the supernatant of cell culture medium from human colon carcinoma cell line WiDR (ATCC No. CCL 218) (Rydlander L, Ziegler E, Bergman T, Schöberl E, Steiner G, Bergman A-C, Zetterberg A, Marberger M, Björklund P, Skern T, Einarsson R and Jörmvall H (1996): Molecular characterization of a tissue-polypeptide-specific-antigen epitope and its relationship to human cytokeratin 18. Eur.J.Biochem. 241:309-314.) The first step utilized precipitation with 50% (mass/vol) ammonium sulfate. This was followed by hydrophobic-interaction chromatography on phenyl Sepharose in 14 mM phosphate/85 mM ammonium sulfate, pH 7.5. After washing of the column, hydrophobic proteins were eluted with water, and fractions collected. The third step consisted of Sephacryl S-300 exclusion chromatography in 8 M urea, 0.1 M Tris/HCl, pH 8.0. In the fourth step, Q Sepharose, equilibrated with 8 M urea in 0.1 M Tris/HCL, pH 8.0. was employed.

25

The fraction eluted at 0.12 M NaCl was about 882 times purified and contained two active components, one of 13 kD, another of 22 kD. They were identified as subtypes of cytokeratin 18, both ending at aa 396, i.e. fragments comprising a C-terminal aa sequence ALD.

30

Immunization procedure

Four BalbC mice received one intraperitoneal (i.p.) injection of 100 µl of Freund's complete adjuvant suspended in 100 µl balanced salt solution (PBS)

containing 107 µg of the first batch of the purified antigen. Three, six and nine weeks later, the mice received similar injections and amounts of an additional batch of the antigen, but with incomplete instead of complete Freund's adjuvant. Eight weeks after the last injection, a booster injection of 53 µg of a new batch of antigen without adjuvant in 100 µl PBS was given i.v. and the same amount was introduced i.p.

Immunization of BalbC mice with an appropriate dose of the synthesized peptide **EDFNLGDALD**, coupled to the lipopeptide adjuvant: (Pam3cysSer(Lys)₄, Boehringer 1428 764, lot 1312 7024), will result in development of immuno-competent cells producing monoclonal antibodies against the synthetic oligopeptide.

Hybridization

Three days after the booster injection of the mice, the spleen cells were hybridized with mouse myeloma cells (cell line: P3x63-Ag 8.653, G.Köhler)). Mediums from a large number of resulting hybridoma cultures were reacted with rabbit anti-mouse antibodies (solid phase), whereupon immunizing antigen and blocking mouse globulin were added, followed by HRP-labeled, detecting antibody against CK 18.

By immunocytochemical analyses it was observed that one of the resulting hybrid cell lines (No. 30) produced antibodies that reacted with cultured cells, that were neither vital nor necrotic. Among the cells tested were HeLa cells (Gey 1952), bladder carcinoma cell line T24, mammary cell line T47d, colon carcinoma cell line WiDr CCL 218 grown in IMDM 90% + 10% fetal bovine serum + 2 mm L-glutamine and 10 µg Gentamycine. The unexpected observations gave rise to detailed studies of the antibody specificity by immunochemical and immunocytochemical techniques. It became evident that the MAb, designated M30, reacted specifically with apoptotic cells and fluids.

Immunoch mistry

In order to characterize the MAb M30-epitope chemically, the M30 was tested against a large number of synthetic peptides, that were especially made for this purpose. It was found that the optimal specific epitope consisted of the aa
 5 sequence EDFNLGDALD. This and related sequences are presented in Table 1 which shows the reactivity of amino-acid sequences with the monoclonal antibody M 30 of the invention.

Table 1

10

| Peptide No. | Link to Biotin | Absorbance at 405/492 nm | Sequence |
|-------------|----------------|-----------------------------|-----------------|
| 63 | -SGSG- | 0.05 | LEDGEDFNLGDAL |
| 15 61 | -SGSG- | 0.1 | LEDGEDFNLGDALDS |
| 46 | -SGSG- | 0.77 | DGEDFNLGDALD |
| 45 | -SGSG- | 1.05 | EDGEDFNLGDALD |
| 47 | -SGSG- | 1.23 | GEDFNLGDALD |
| 302 | -SGSB- | 1.41 | GEDFNLGDALD |
| 20 301 | -SGSB- | 1.46 | EDFNLGDALD |
| 401 | -SGSB- | 0.62 | DFNLGDALD |
| 402 | -SGSB- | 0.27 | FNLGDALD |
| 403 | -SGSB- | 0.14 | NLGDALD |
| 404 | -SGSB- | 0.1 | LGDALD |
| 25 405 | -SGSB- | 0.1 | GDALD |
| 406 | -SGSB- | 0.1 | DALD |

Note. B = β alanine

30 As is evident from Table 1, gradual shortening of the sequence from the amino end to EDFNLGDALD, gave maximal activity. At the other end, removal of the last D (Asp) or addition of an S (Ser) to the last D resulted in complete loss of activity.

The need for the last D in DALD is clear-cut and should be seen in connection with what is known about structures required for apoptosis: Active caspase-3 enzyme recognizes an DXXD pattern in target substrates (Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SR, Smulson ME, Yamin T-T, Yu VL, Miller DK, et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*. 376:37-43.). In addition, both caspase-6 and caspase-7 are able to cleave substrates with a DXXD sequence (Talanian RV, Quinlan C, Trautz S, Hacket MC, Mankovich JA., Banach D, Ghayur T, Brady KD, Wong WW. (1997). Substrate specificities of caspase family proteases. *J.Biol.Chem.*272:9677-9682.). So far, the new sequence described here has not been known before to indicate apoptosis.

That proteases of the caspase subfamily are involved in mammalian apoptosis is supported by compelling evidence ranging from observation of DXXD-X cleaving activity and the activation of proenzymes in apoptotic cells, and the inhibition of apoptosis in a variety of systems by specific substrate-mimicking peptide inhibitors (Nobel S.(1997). *Thiol redox state in apoptosis: Physiological and toxicant modulation*. Dissertation, Karolinska Institutet, Stockholm, Sweden. ISBN 91-628-2502-X.).

Immunocytochemistry

Today there are several methods for the detection of apoptosis, but none is based on a defined reacting part of cytokeratin 18 with known amino acid sequence. The most widely used methods to identify apoptotic cells are light and electron microscopic analysis, flow cytometry, agarose gel electrophoresis, in situ nick-end labeling (ISEL) and the TdT-mediated dUTP Nick End Labeling (TUNEL)-technique.

30

To substantiate the preliminary findings, and to fit the expression of the epitope, recognized by the MAb M30, the effect of the different apoptosis systems were analyzed and a comparison made between M30-immunocytochemistry and

accepted apoptosis assays. Comparisons were made using e.g. the non small cell lung cancer cell line MR65 with the annexin-, the TUNEL- and flow cytometric assays next to established morphological criteria. Confocal laser microscopic analysis revealed that apoptotic cells showed a bright
5 immunofluorescence cytoplasmic staining after incubation with the MAb M30, while viable and necrotic cells turned out to be negative.

The expression of the M30-epitope correlated very well with other accepted staining methods for the detection of apoptotic cells i.e. annexin V-, TdT-mediated dUTP Nick End Labeling (TUNEL)-assay and morphological criteria.
10 The majority of the M30-positive cells were in the "apoptotic" sub G1-peak. The expression of the M30-epitope occurred early in the apoptotic cascade, before annexin V reactivity or positive Nick end labeling. The epitope was not detectable in vital epithelial cells. The epitope, consisting of the aa sequence
15 EDFNLGDALD or functionally equivalent sequences comprising at least the sequence Ala Leu Asp, allows quantification of apoptotic cells at the level of the cytoskeleton using simple immunocytochemical techniques.

The major drawbacks of the hitherto accepted methods for detection of
20 apoptosis are that they are time consuming, relatively expensive, labor-intensive and not always specific for apoptotic cells. In addition, the TUNEL assay and the ISEL can only detect intermediate and late apoptosis.

When the effects of different apoptosis induction systems were analyzed and a
25 comparison made between M30-immunocytochemistry and accepted apoptosis-assays, it turned out, that M30 is superior to existing apoptosis detection assays in cell lines and routinely obtained biopsies of human origin. M30 specifically recognizes apoptotic cells in the early stages of the process.

30 Since M30 recognizes the intrinsic early marker of apoptosis, i.e. EDFNLGDALD or functionally equivalent sequences comprising at least the sequence ALD, it is applicable to fresh and also formalin fixed, paraffin embedded tissue sections of routinely obtained biopsies. This is one of the advantages of M30.

Since M30-immunoreactivity also precedes both loss of membrane asymmetry, as is detected by annexin V-binding and DNA fragmentation, which is detected by the TUNEL assay, it offers distinctive advantages over presently used routine assays. Since apoptosis is implicated in many diseases, the specific, early detection and quantification of apoptotic cells is of utmost importance.

Body fluids reflecting apoptosis

When apoptotic products containing EDFNLGDALD, or functionally equivalent sequences comprising at least the sequence ALD, are released from apoptotic cellular areas in the body into body fluids such as serum, ascites, pleura, etc., immunoreactive assays can be used with MAb M30 just as with other serum assays to detect and quantify the concentration of special products in serum and body fluids. In this way one can differentiate between illnesses caused by or connected with enhanced or insufficient apoptosis i.e. increased or decreased levels of the MAb M30 binding site EDFNLGDALD or functionally equivalent sequences comprising at least the sequence ALD. Specimens from implicated organs or tissues can contribute to a more specific diagnosis.

20 Diagnostic use of the SEQ ID NO: 1 or functionally equivalent sequences

The process of apoptosis can be subdivided into three phases: the induction phase, the effector phase and the degradation phase. Cytokeratin 18 degradation and production of fragments containing EDFNLGDALD takes place very early and long before any of the known methods for assay of apoptosis turn positive. This makes it possible to interfere very early with the production and function of EDFNLGDALD, or functionally equivalent sequences comprising at least the sequence ALD, with the aid of antagonistic medicals, specific antibodies to EDFNLGDALD, or functionally equivalent sequences comprising at least the sequence ALD, and also inhibitors or activators of the enzymes that contribute to the production of EDFNLGDALD or functionally equivalent sequences comprising at least the sequence ALD.

By using the knowledge obtained from the analysis of concentration and localization of M30-positive specimens, it is possible to tell, if enhancement or inhibition of the process of apoptosis (programmed cell death) is desirable and if one should try to influence this process by labeled or unlabeled M30-antibody, more labeled or unlabeled EDFNLGDALD, or functionally equivalent sequences comprising at least the sequence ALD, labeled or unlabeled antagonist to EDFNLGDALD, functionally equivalent sequences comprising at least the sequence ALD, such as KRWQFGRLAR or similar, or by inhibiting or enhancing the enzyme producing EDFNLGDALD or functionally equivalent sequences comprising at least the sequence ALD. The choice is based upon the exact determination of the apoptotic amino acid sequence EDFNLGDALD or functionally equivalent sequences comprising at least the sequence ALD with the aid of the monoclonal antibody M30.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

(A) NAME: Beki AB (Publ.)

(B) STREET: Fredsforsstigen 22

(C) CITY: Bromma

10

(E) COUNTRY: Sweden

(F) POSTAL CODE (ZIP): 168 66

(G) TELEPHONE: 08-290370

(ii) TITLE OF INVENTION: Apoptosis-related compounds and their use

15

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

20

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

30

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40

Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp

1

5

10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

15

Glu Asp Gly Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp Ser Ser Asn Ser
1 5 10 15

20 Met Gln Thr
20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

30

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

35

Leu Glu Asp Gly Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp Ser Ser
1 5 10 15

40 Asn Ser

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gly Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp
1 5 10

CLAIMS

1. Apoptosis-related antigenic compound comprising an exposed antigenic site having the amino acid sequence
- 5 SEQ ID NO: 1:
- Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp
- 1 5 10
- or a functionally equivalent sequence comprising at least the sequence Ala Leu Asp.
- 10
2. Antigenic compound according to claim 1, wherein the compound is a peptide, or a protein fragment comprising the C-terminal amino acid sequence Ala Leu Asp.
- 15
3. Antigenic compound according to claim 2, wherein the compound is a peptide or a protein fragment comprising the C-terminal amino acid sequence Xaa Ala Leu Asp, wherein Xaa is selected from Asp, Leu, Met, and Val.
- 20
4. Antigenic compound according to claim 2 or 3, wherein the peptide is an oligopeptide having the amino-acid sequence
- SEQ ID NO: 1:
- Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp
- 1 5 10
- 25
5. Antigenic compound according to claim 2 or 3, wherein the peptide is an oligopeptide having the amino-acid sequence
- SEQ ID NO: 4:
- Gly Glu Asp Phe Asn Leu Gly Asp Ala Leu Asp.
- 30 1 5 10
6. Antigenic compound according to any one of claims 1 - 5 coupled to a carrier and/or label.

7. Antigenic compound according to any one of claims 1 - 6 for use in a medicament.
- 5 8. Antigenic compound according to claim 7 for use in the production of a medicament for inhibition of cell apoptosis.
9. Isolated or recombinant nucleic acid sequence comprising a nucleotide sequence which encodes an amino-acid sequence of a peptide or protein
- 10 10. Isolated or recombinant nucleic acid sequence comprising a nucleotide sequence which encodes an amino-acid sequence of a peptide or protein fragment according to any one of claims 2 - 6.
10. Anti-sense nucleic acid sequence based on a sense nucleic acid sequence according to claim 9.
- 15 11. Vector comprising a nucleic acid sequence according to claim 9 or 10.
12. Vector according to claim 11, wherein the vector is a plasmid.
13. Antibody or antigen-binding peptide recognizing an antigenic compound
- 20 14. Antibody or antigen-binding peptide according to claim 13 coupled to a carrier and/or label.
15. Antibody or antigen-binding peptide according to claim 13 or 14 for use in a medicament.
16. Antibody or antigen-binding peptide according to claim 15 for use in the production of a medicament for the stimulation of cell apoptosis.
- 30 17. Agent regulating the liberation in biological material, including the mammalian body or cell culture, of protein fragments comprising the C-terminal amino-acid sequence Ala Leu Asp.

18. Method of determining the occurrence of cell apoptosis in a biological sample including a sample of an organ, tissue or body fluid from a mammal, including man, wherein the presence of protein fragments comprising the
5 C-terminal amino-acid sequence
Ala Leu Asp
is determined.
19. Method according to claim 18 , wherein the determination is performed with
10 an immunological assay using the antibody of claim 13 or 14.
20. Method according to claim 18 or 19, wherein the rate of occurrence of cell apoptosis is determined.
- 15 21. Method according to claim 20, wherein the determined rate of cell apoptosis is used in the diagnosis of diseases with involvement of apoptosis, such as degenerative diseases and cancer and/or monitoring of the effect of therapy.
22. Method of treating diseases with involvement of apoptosis, such as cancer
20 and degenerative diseases, in a patient comprising administration of a cell apoptosis-regulating amount of an antibody or antigen-binding peptide according to any one of claims 13 -16, or of an antigenic compound according to any one of claims 1 - 6, to said patient.
- 25 23. Method of creating cancer cells comprising administration of a cell apoptosis-inhibiting amount of an antigenic compound according to any one of claims 1-6 to a cell culture or a laboratory animal.
24. Carrier for prophylactic, therapeutic or diagnostic use comprising an
30 antibody or antigen-binding peptide recognizing an antigenic compound according to any one of claims 1 - 6.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01721

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/47, C07K 16/18, A61K 38/39 // A61K 38/08
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| X | The journal of Cell Biology, Volume 138, No 6, Sept 1997, Carlos Caulín et al, "Caspase Cleavage of Keratin 18 and Reorganization of Intermediate Filaments during Epithelial Cell Apoptosis", page 1379 - page 1393, page 1391; column 1, line 46 - line 51, figures | 1-6,9-15, 18-21 |
| Y | | 7,24 |
| A | | 8,16,23 |
| | -- | |
| Y | WO 9205197 A1 (SILEN, AKE), 2 April 1992. (02.04.92), claim 13 | 7,24 |
| A | | 8,16,23 |
| | -- | |

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 January 1999

Date of mailing of the international search report

22 January 1999

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01721

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| P,X | STN International, file Scisearch, Scisearch accession no. 1998:40215, Ku N O et al: "Apoptosis generates stable fragments of human type I keratins"; Journal of Biological Chemistry, (26 DEC 1997) Vol. 272, No. 52, pp. 33197-33203 -- ----- | 1-16,18-21, 23-24 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01721

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 22
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods
2. ☒ Claims Nos.: 17
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see next sheet
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of an additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01721

See PCT art 6. As the claimed compound having at least the sequence ALD relates to a marker early in the development of apoptosis, the agent in claim 17 could be any substance causing apoptosis directly or indirectly. There are potentially an immense number of such agents. Thus, claim 17 is not considered to be clear and concise and consequently, does not fulfil the requirements of PCT art 6.

01/12/98

PCT/SE 98/01721

Form PCT/ISA/210 (patent family annex) (July 1992)